See related commentary on pg 1209

Agents that Reverse UV-Induced Immune Suppression and Photocarcinogenesis Affect DNA Repair

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UV exposure induces skin cancer, in part, by inducing immune suppression. Repairing DNA damage, neutralizing the activity of *cis*-urocanic acid, and reversing oxidative stress abrogate UV-induced immune suppression and skin cancer induction, suggesting that DNA, UCA, and lipid photo-oxidation serve as UV photoreceptors. What is not clear is whether signaling through each of these different photoreceptors activates independent pathways to induce biological effects or whether there is a common checkpoint where these pathways converge. Here, we show that agents known to reverse photocarcinogenesis and photoimmune suppression, such as platelet-activating factor (PAF) and serotonin (5-HT) receptor antagonists, regulate DNA repair. Pyrimidine dimer repair was accelerated in UV-irradiated mice injected with PAF and 5-HT receptor antagonists. Nucleotide excision repair (NER), as measured by unscheduled DNA synthesis, was accelerated by PAF and 5-HT receptor antagonists. Injecting PAF and 5-HT receptor antagonists into UV-irradiated *Xeroderma pigmentosum* complementation group A–deficient mice, which lack the enzymes responsible for NER, did not accelerate photoproduct repair. Similarly, UV-induced formation of 8-oxo-deoxyguanosine was reduced by PAF and 5-HT receptor antagonists. We conclude that PAF and 5-HT receptor antagonists accelerate DNA repair caused by UV radiation, which prevents immune suppression and interferes with photocarcinogenesis.

Journal of Investigative Dermatology (2010) **130,** 1428–1437; doi:10.1038/jid.2009.329; published online 15 October 2009

INTRODUCTION

The UV radiation found in sunlight is the primary cause of non-melanoma skin cancer, the most prevalent human cancer, and UV exposure is implicated in the induction of melanoma, the most dangerous skin cancer (Boring *et al.*, 1992). In addition to being a complete carcinogen, UV is immune suppressive, and the induction of immune suppression by UV exposure is recognized as a major risk factor for skin cancer induction (Fisher and Kripke, 1982; Yoshikawa *et al.*, 1990). UV irradiation induces signature mutations (C to T and CC to TT transitions) in the *p53* tumor suppressor gene, a critical target for skin cancer formation (reviewed by Benjamin *et al.*, 2008). Further, UV-induced DNA lesions, particularly cyclobutane pyrimidine dimer (CPD) formation, have an essential role in the induction of immune suppression (Applegate *et al.*, 1989; Vink *et al.*, 1997), identifying DNA as an important photoreceptor for UV-induced carcinogenesis and immunosuppression.

DNA is not alone in its ability to act as a photoreceptor for UV-induced immunosuppression. The isomerization of *trans*-UCA to the *cis*-isomer converts UV radiation into a biologically recognizable signal that activates immune suppression (De Fabo and Noonan, 1983). Neutralizing the activity of *cis*-urocanic acid (*cis*-UCA) (Moodycliffe *et al.*, 1996), or blocking the binding of *cis*-UCA to its receptor (5-HT_{2A}) with selective serotonin receptor antagonists, blocks UV-induced immune suppression (Walterscheid *et al.*, 2006). Neutralizing the activity of *cis*-UCA *in vivo* blocks photocarcinogenesis (Beissert *et al.*, 2001).

Evidence from a number of laboratories has also indicated that membrane phospholipids can absorb UV radiation, leading to lipid peroxidation and the activation of transcription factors, resulting in cytokine release and contributing to immune suppression (Devary *et al.*, 1993; Simon *et al.*,

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Abbreviations: 1-NPZ, 1-(1-naphthyl)piperazine; 5-HT, serotonin; 6–4 photoproducts, pyrimidine (6–4) pyrimidinone photoproducts; 8-oxo-dG, 8-oxo-deoxyguanosine; cPAF, carbamyl-PAF; cis*-UCA,* cis*-urocanic acid; CPD, cyclobutane pyrimidine dimer; NER, nucleotide excision repair; PAF, platelet-activating factor; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; UDS, unscheduled DNA synthesis; UVB, ultraviolet light B; XPA, Xeroderma pigmentosum complementation group A*

Received 14 July 2009; revised 31 August 2009; accepted 9 September 2009; published online 15 October 2009

1994). Moreover, injecting UV-irradiated phospholipids into mice activates cytokine release and immune suppression, further supporting a role for lipids as a photoreceptor (Walterscheid *et al.*, 2006). The lipid mediator of inflammation, platelet-activating factor (PAF), is secreted by keratinocytes almost immediately after UV exposure (Pei *et al.*, 1998), and activates the transcription of many of the cytokines and immunomodulatory factors, such as IL-10 and prostaglandin E_2, which drive UV-induced immune suppression. Blocking the binding of PAF to its receptor blocks UV-induced immune suppression (Walterscheid *et al.*, 2002). Moreover, UV exposure promotes oxidative stress, which induces DNA damage, and the production of oxidized phospholipids, such as PAF, and treating UV-irradiated mice with antioxidants blocks UV-induced immune suppression and photocarcinogenesis (Black *et al.*, 1997).

What is not clear is whether signaling through each of these different photoreceptors activates independent pathways to induce immune suppression and photocarcinogenesis or whether there is a common checkpoint where these pathways converge. Recently, we showed that PAF and 5-HT receptor antagonists block photocarcinogenesis (Sreevidya *et al.*, 2008). To determine, in more detail, the mechanisms involved, we measured the numbers of CPDs in the skin of antagonist-treated UV-irradiated mice. Initially, we noted that the numbers of CPDs in the skin of the antagonist-treated, UV-irradiated mice were similar to those seen in the UV-only controls. However, as time progressed, UV-induced CPDs were repaired more rapidly in the receptor antagonist-treated mice. In view of the importance that CPD formation has in both carcinogenesis and immune suppression, we asked whether the mechanism by which diverse agents such as PAF and 5-HT receptor antagonists reverse immune suppression and carcinogenesis is by regulating DNA repair. Here, we present data showing that blocking PAF and *cis*-UCA from binding to their receptors modulates DNA repair following exposure to UV radiation.

RESULTS

Agents that reverse immune suppression accelerate photoproduct removal

Over the years, a variety of structurally and mechanistically diverse agents have been used to overcome UV-induced immune suppression. This is illustrated in Figure 1. Repairing CPD formation (Figure 1a) by applying T4N5-containing liposomes (Kripke *et al.*, 1992), neutralizing the activity of *cis*-UCA (Figure 1b) (El-Ghorr and Norval, 1995), blocking PAF receptor binding (Figure 1c) (Walterscheid *et al.*, 2002), and blocking 5-HT receptor binding (Walterscheid *et al.*, 2006) (Figure 1d), all interfere with UV-induced immune suppression. Alternatively, treating mice with PAF, 5-HT, and *cis*-UCA all activate immune suppression. We measured the effect these agents had on the numbers of CPD and pyrimidine (6–4) pyrimidinone photoproduct (6–4 photoproduct) in the skin. Mice were exposed to 2 kJ m^{-2} of ultraviolet B (UVB) radiation and immediately injected with a PAF receptor antagonist (PCA-4248), or a 5-HT receptor antagonist 1-(1-naphthyl)piperazine (1-NPZ), as previously described (Walterscheid *et al.*, 2006; Sreevidya *et al.*, 2008). At various times post irradiation, skin samples were acquired; DNA was isolated and CPD and 6–4 photoproduct numbers were determined by immunohistochemistry (Figure 1e and f) and ELISA (Figure 1g and h). Note that at 1 hour post irradiation, the numbers of CPDs and 6–4 photoproducts found in the skin of the mice injected with the receptor antagonists were similar to the numbers found in mice only exposed to UV radiation. At 12 and 24 hours post irradiation, we saw a significant ($P<0.05$) decrease in the numbers of CPD and 6–4 photoproducts found in the skin of UV-irradiated and PAF- and/or 5-HT-antagonist-injected mice, compared with the UV-irradiated control. By 48 hours, no significant difference in the numbers of CPD and 6–4 photoproducts found in the skin of the UV-irradiated and PAF- and/or 5-HT-injected mice, *versus* the number of CPD and 6–4 photoproducts found in the skin of the UV-only controls, was noted. These data indicate that treating UV-irradiated mice with PAF and 5-HT receptor antagonists accelerates the repair of UV-induced photoproducts.

Effect of PAF and 5-HT receptor antagonists on unscheduled DNA repair

UV-induced photoproducts are removed by nucleotide excision repair (NER), which is routinely detected by unscheduled DNA synthesis (UDS) (Hanawalt *et al.*, 2003). Therefore, we asked whether accelerated photoproduct repair was due to NER. Keratinocytes were pretreated with a PAF (PCA-4248, 50 μM) or a 5-HT (1-NPZ; 25 μM) receptor antagonist, washed, and then UV-irradiated (200 J m^{-2}). The cells were incubated with BrdU and then stained with anti-BrdU antibody (Figure 2a). The left panel shows anti-BrdU staining, the middle panel shows DAPI (4′-6-diamidino-2-phenylindole) staining to illuminate the nuclei, and the right panel shows a merge of the DAPI and BrdU panels. Compared with unirradiated control cells, punctate nuclear staining (arrows), indicative of UDS, was found in cells exposed to UV radiation (Figure 2a). Treating the cells with the PAF or the 5-HT receptor antagonist further increased the number of cells undergoing UDS (35.4% UV only *vs* 55.4% UV + PCA 4248, and *vs* 58.8% UV + 1-NPZ). In addition, the number of grains per nuclei was counted and we found that treating the cells with the PAF or 5-HT receptor antagonists increased the grains per nuclei (Figure 2b). These data indicate that PAF and 5-HT receptor antagonists accelerate NER.

Failure of PAF and 5HT receptor antagonists to accelerate DNA repair in *XPA*-deficient mice

NER is absent in *Xeroderma pigmentosum* complementation group A (*XPA*−/−) mice (Hanawalt, 2001). To confirm that NER is involved in the accelerated DNA repair induced by PAF and 5-HT receptor antagonists, we measured CPD removal in *XPA*−/− mice. Wild-type and *XPA*−/− mice were UV-irradiated and then injected with PAF and 5-HT receptor antagonists. CPD was observed in wild-type mice 24 hours after UV irradiation. Treating the wild-type mice with a PAF and/or a 5-HT receptor antagonist accelerated the rate of DNA

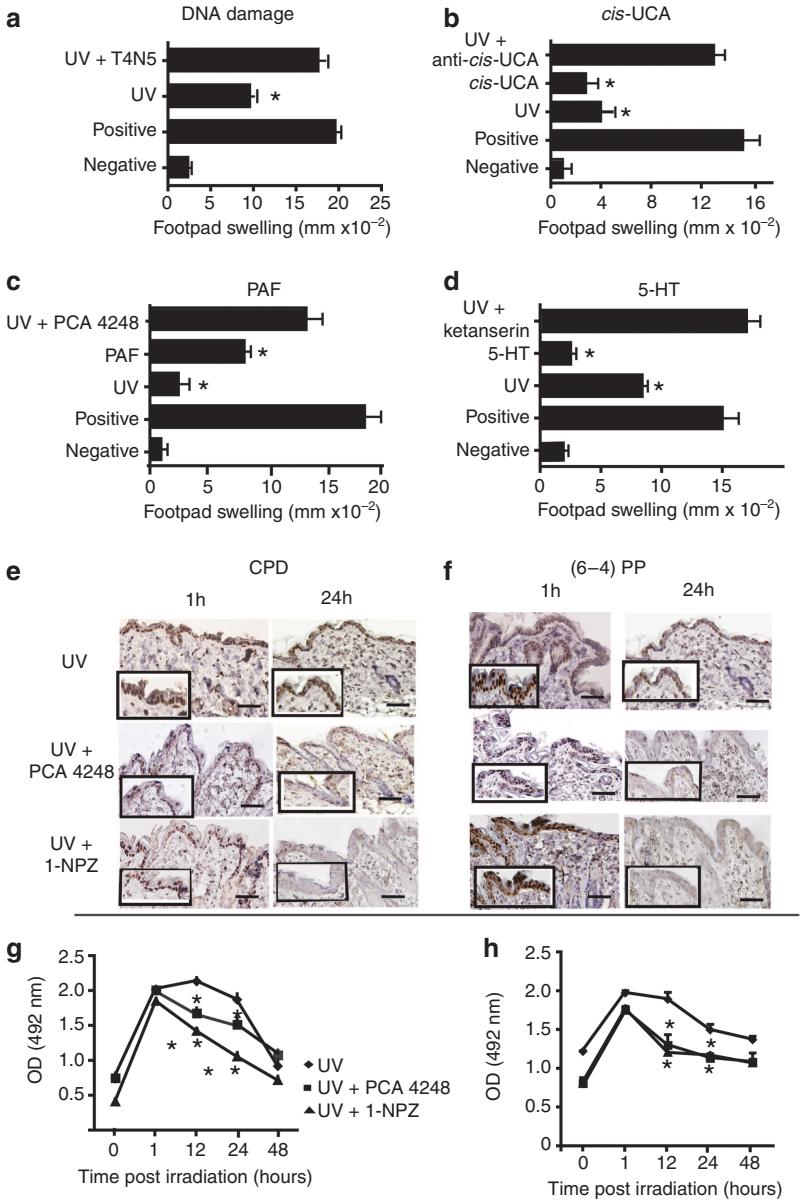

Figure 1. Reversal of immune suppression and acceleration of DNA repair by platelet-activating factor (PAF) and serotonin (5-HT) receptor antagonists. Effect of agents that repair DNA damage, neutralize *cis*-urocanic acid (*cis*-UCA) activity, or block PAF and 5-HT from binding to their receptors on immune suppression. Mice were exposed to 15 kJ m^{-2} of ultraviolet B (UVB) radiation and then treated with (**a**) T4N5-containing liposomes, (**b**) monoclonal anti-*cis*-UCA, (**c**) PAF receptor antagonist, and (**d**) 5-HT receptor antagonist. The effect the various treatments had on UV-induced immune suppression was determined using a delayed-type hypersensitivity (DTH) assay. The data are expressed as mean footpad swelling ± SEM. The asterisk denotes a significant difference ($P<0.05$) from the positive control. Effect of PAF and 5-HT receptor antagonists on the numbers of cyclobutane pyrimidine dimmer (CPD) and pyrimidine (6–4) pyrimidinone photoproducts (6–4 photoproducts). Mice were exposed to 2 kJ m^{-2} of UVB radiation and then immediately injected with 1 μM of PCA-4248 or 1 μM of 1-(1-naphthyl)piperazine (1-NPZ). At 1 and 24 hours post irradiation, skin samples were removed, and (**e**) CPD or (**f**) 6–4 photoproducts (PP) were visualized by immunohistochemistry. Bar = 50 μm. High-magnification views (× 400) are shown in the inserts. At various times post irradiation, skin samples were taken, DNA was isolated, and (**g**) CDP and (**h**) 6–4 photoproducts were measured by ELISA. *Indicates a significant difference ($P<0.05$) from the UV-only control (Student's *t*-test, $n=5$).

repair (Figure 3a). No removal of CPD was noted when the PAF and/or the 5-HT receptor antagonists were injected into UV-irradiated *XPA*−/− mice. In addition, we saw no inhibition of apoptosis in *XPA*−/− mice injected with PAF or 5-HT receptor antagonists (Figure 3b). These findings support our hypothesis that PAF and 5-HT receptor antagonists accelerate the rate of DNA repair through a mechanism involving NER.

Induction of ROS and DNA damage following treatment with PAF, 5-HT, and *cis*-UCA

UV induces oxidative stress, which can lead to free radical formation and ultimately DNA damage (Bertram and Hass, 2008). Although PAF and 5-HT have been shown to activate reactive oxygen species (ROS) and apoptosis (Barber *et al.*, 1998; Breard *et al.*, 2007), it is not clear whether *cis*-UCA can

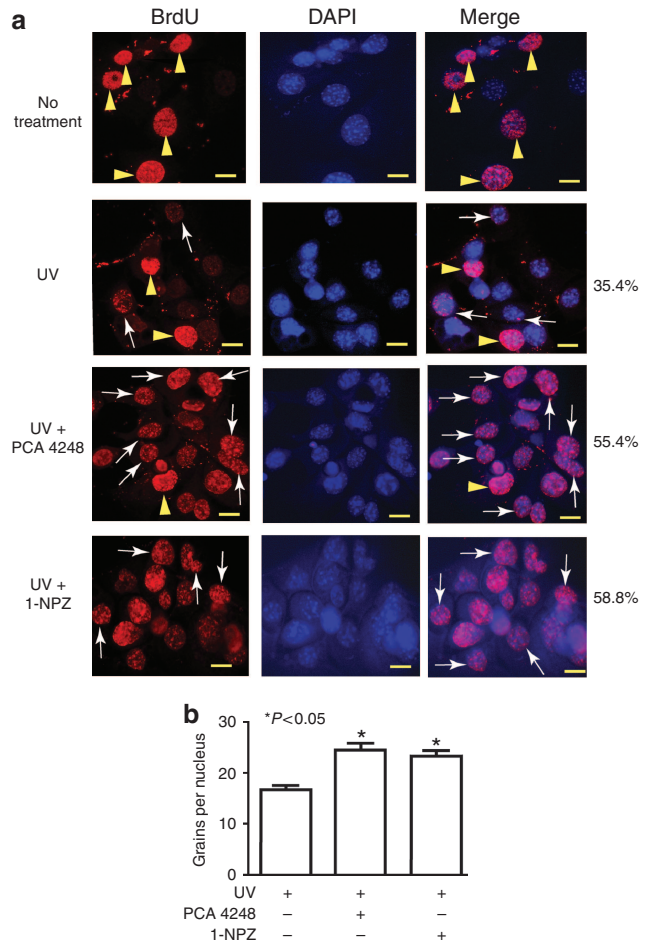

Figure 2. Unscheduled DNA synthesis is accelerated by treating UV-irradiated mice with PAF and serotonin (5-HT) receptor antagonists. (**a**) Pam 212 cells were treated with a PAF (PCA-4248; 50 μM) or a 5-HT (1-(1-naphthyl)piperazine (1-NPZ); 25 μM) receptor antagonist, washed, and then exposed to 200 J m^{-2} of ultraviolet B (UVB) radiation. The cells were incubated with BrdU (3 hours, 37°C) and then incubated with anti-BrdU antibody. The left panels show anti-BrdU staining (red). The center panels show 4′-6-diamidino-2-phenylindole (DAPI) staining of the nuclei. The right panel shows a merge of the BrdU and DAPI staining. Arrowheads illustrate nuclei in the S phase; arrows indicate punctate nuclear staining characteristic of cells undergoing unscheduled DNA synthesis (UDS). The percentage of cells undergoing UDS in each group is indicated. Bar = 50 μm. (**b**) Quantification of UDS. The number of individual grains per nuclei was counted. *Indicates a significant difference ($P<0.05$) from the UV-only control (Student's *t*-test, 50 cells per sample).

induce ROS and promote DNA damage. To address this question, keratinocytes were treated with UV, carbamyl-PAF (cPAF) (the metabolically stable analog of PAF), 5-HT, and *cis*-UCA. The cells were then incubated with 2′,7′-dichlorodihydro-fluorescein diacetate, a dye that is oxidized into the highly fluorescent product dichlorofluorescein by ROS (Brubacher and Bols, 2001). Fluorescence was measured by flow cytometry. The positive controls (UV radiation, cPAF, and 5-HT) activated ROS production as measured by the increased numbers of highly fluorescent cells (Figure 4a). Treatment with *cis*-UCA also induced ROS formation.

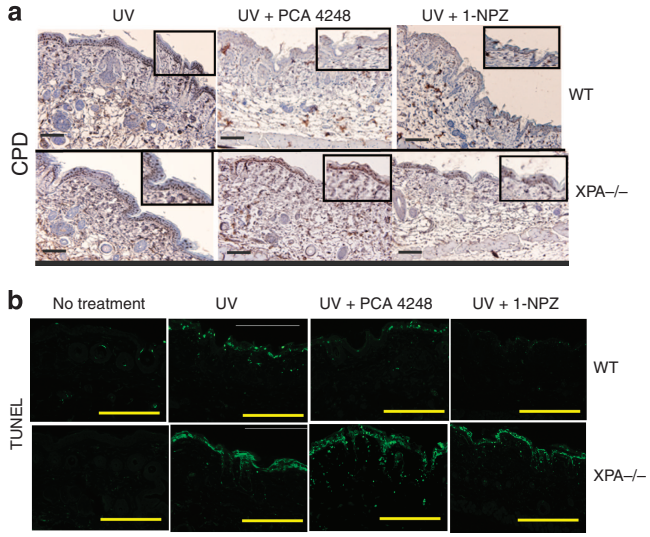

Figure 3. Failure to reverse cyclobutane pyrimidine dimmer (CPD) formation in PAF and serotonin (5-HT) receptor antagonist-treated *XPA*−/− mice. Wild-type (WT) or *XPA*−/− mice were exposed to 2 kJ m^{-2} of ultraviolet B (UVB) radiation and immediately injected with 1 μM of PCA-4248 or 1 μM of 1-(1-naphthyl)piperazine (1-NPZ). (**a**) CPDs were detected by immunohistochemistry. Bar = 50 μm. High-magnification views (× 400) are shown in the inserts. (**b**) Apoptosis was detected by TUNEL. Bar = 200 μm.

Treating the cells with PCA-4248, or 1-NPZ, reduced the number of intensely staining fluorescent cells, indicating that these agents block ROS production. This observation was confirmed by fluorescent microscopy (Figure 4b). No fluorescence was found in normal cells, but many fluorescent cells were found following UV and/or cPAF exposure. Keratinocytes treated with 5-HT or *cis*-UCA also emitted a strong fluorescent signal. Treating the cells with PAF and 5-HT receptor antagonists reduced the fluorescent signal, supporting the conclusion that PAF, 5-HT, and *cis*-UCA activate ROS production.

ROS induces 8-oxo-deoxyguanosine (8-oxo-dG), which is pre-mutagenic and has been implicated in photocarcinogenesis (Le Page *et al.*, 1995). We measured 8-oxo-dG in the skin of mice injected with PAF and *cis*-UCA (Figure 5a). Background levels of 8-oxo-dG were observed in samples from untreated mice, which is expected because cells express 8-oxo-dG under normal physiological conditions (Wiseman and Halliwell, 1996). UV irradiation increased 8-oxo-dG formation. Treating the mice with cPAF and *cis*-UCA also increased 8-oxo-dG formation. Treating the mice with PCA-4248 or 1-NZP reduced UV-induced 8-oxo-dG production in the skin. 8-oxo-dG production was measured by ELISA (Figure 5b). We observed a statistically significant induction of 8-oxo-dG by cPAF and *cis*-UCA treatment ($P<0.001$ *vs* normal skin), which was inhibited when the mice were treated with the respective receptor antagonist. As severe DNA damage leads to apoptosis, we also asked whether cPAF and *cis*-UCA induced apoptosis (Figure 5c). A high number of TUNEL-positive apoptotic cells were observed in the skin of the treated mice. As Beneke *et al.* (2000) showed

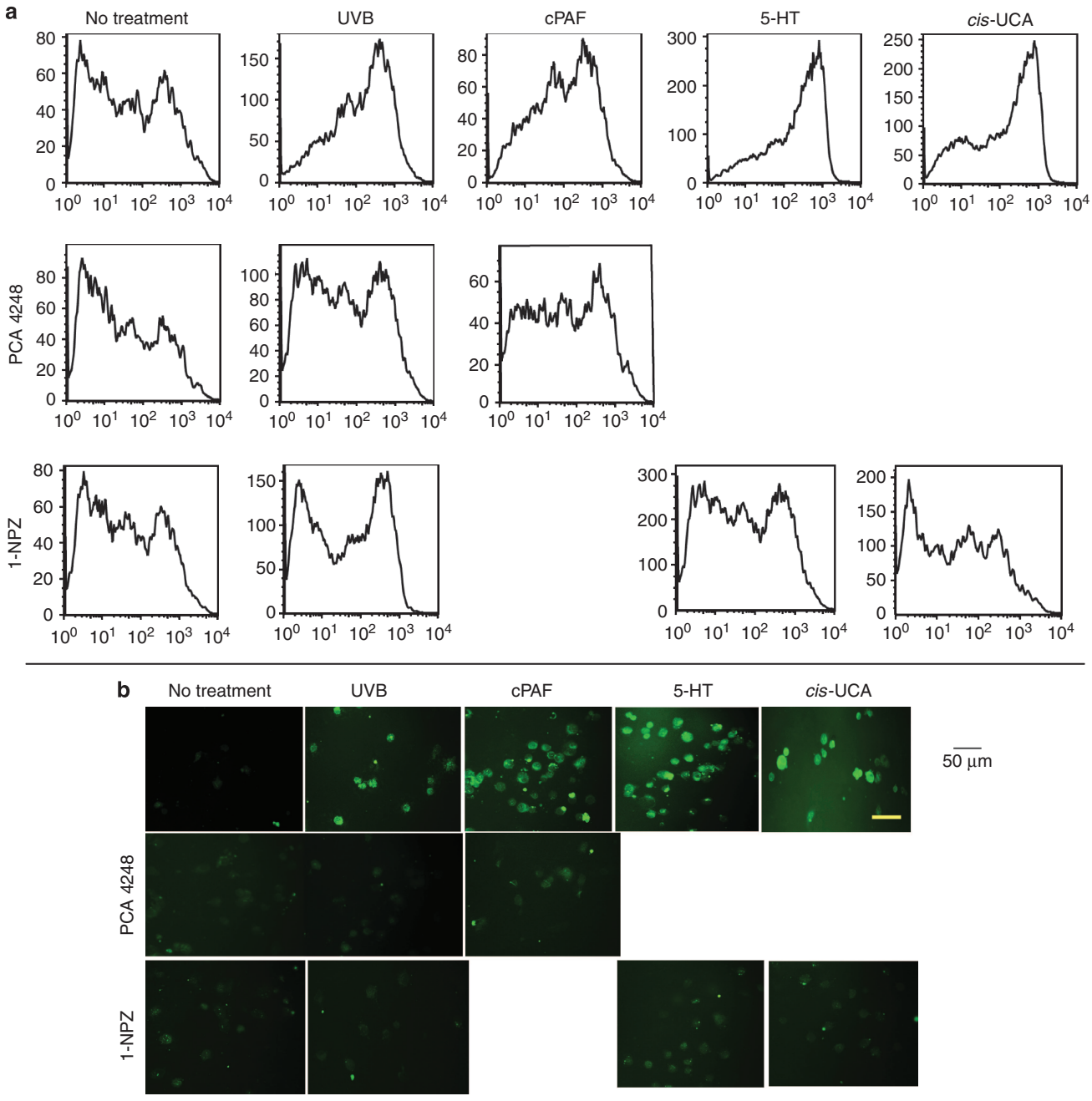

Figure 4. Serotonin (5-HT) and *cis*-urocanic acid (*cis*-UCA) induced reactive oxygen species (ROS) formation. Keratinocytes were exposed to ultraviolet B (UVB) radiation (200 J m^{-2}), or cultured with 10 µM of carbamyl-PAF (cPAF), 50 µM of 5-HT, or 50 µM of *cis*-UCA. Some of the cells were pretreated with PCA-4248 (50 µM) or 1-(1-naphthyl)piperazine (1-NPZ) (25 µM). (**a**) ROS induction was measured by flow cytometry or (**b**) fluorescence microscopy. Bar = 50 µm.

that the cleavage of endogenous Poly (ADP-ribose) polymerase 1 is a marker for apoptotic cell death, we measured the effect of PAF and 5-HT receptor antagonists on poly(ADP-ribose) polymerase (PARP) cleavage. Western blotting with an antibody specific for the cleaved form (89 kDa) of PARP indicated that UV exposure activated PARP cleavage (Figure 5d: lanes 2 and 5). Treating the cells with a PAF (lanes 3 and 6) or a 5-HT (lanes 4 and 7) receptor antagonist inhibited PARP cleavage. These results confirm that both PAF and *cis*-UCA can induce DNA damage, and that blocking the binding of these inflammatory mediators to their receptors will block DNA damage.

DISCUSSION

UV-induced DNA mutations are a critical step in the induction of skin cancer, and compelling evidence from Kripke and colleagues showed that DNA serves as a photoreceptor for UV-induced immune suppression (Applegate

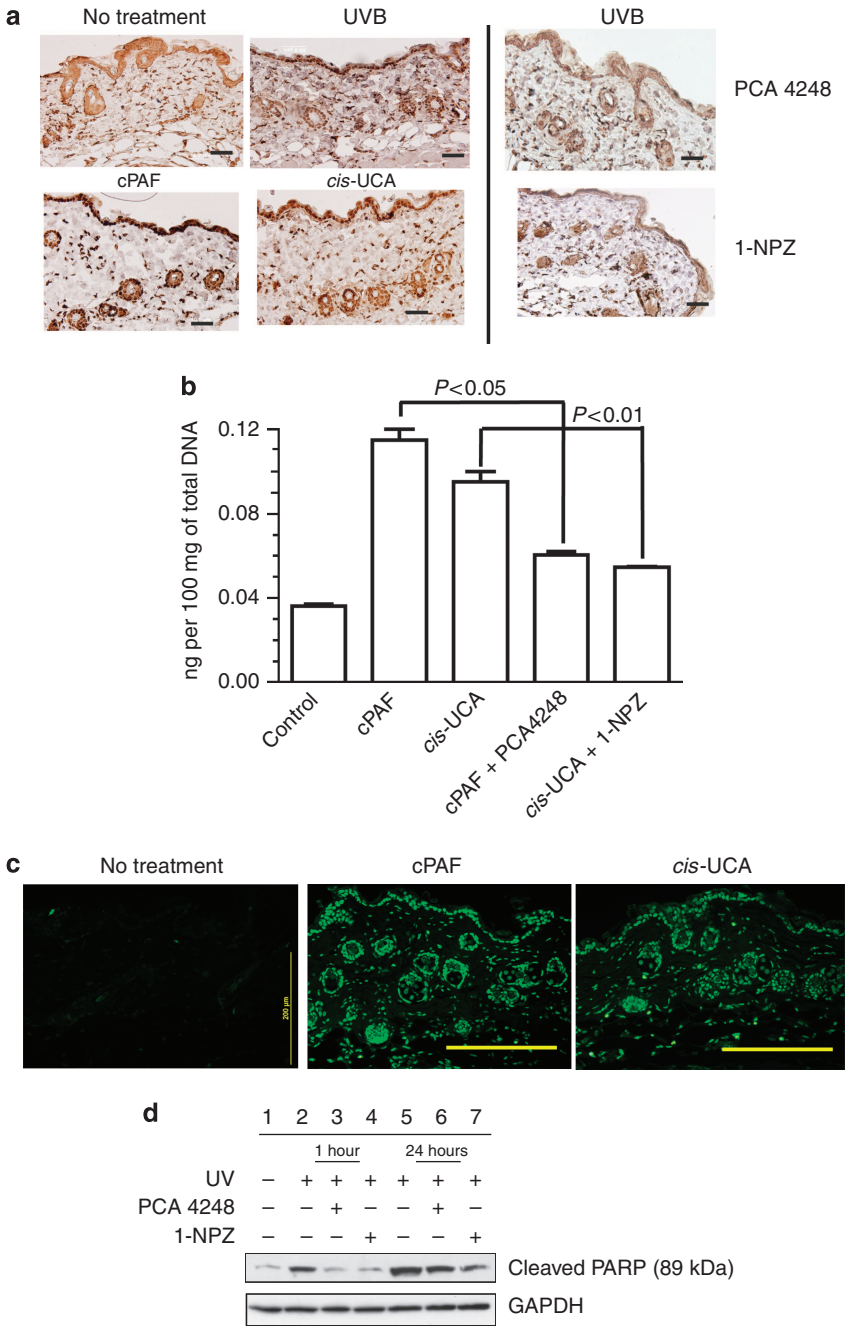

Figure 5. Reduced 8-oxo-deoxyguanosine (8-oxo-dG) lesions following treatment with platelet-activating factor (PAF) and serotonin (5-HT) receptor antagonists. Mice were exposed to 2 kJ m^{-2} of ultraviolet B (UVB) radiation, or injected with 500 nM of carbamyl-PAF (cPAF) or 5 μM of *cis*-urocanic acid (*cis*-UCA). Some mice were injected with 1 μM of PCA-4248 or 1 μM of 1-(1-naphthyl)piperazine (1-NPZ) immediately following irradiation. Skin samples were also collected after dermal injection of 500 nM of cPAF or 5 μM of *cis*-UCA. All samples were collected 24 hours post treatment. (**a**) 8-oxo-dG was detected by immunohistochemistry. (**b**) 8-oxo-dG was measured by ELISA. Data are expressed as ng of 8-oxo-dG per 100 mg total DNA. *P*-values were determined by Student's *t*-test ($n = 5$). (**c**) Apoptosis (TUNEL) in response to dermal cPAF and *cis*-UCA injection. Bar = 200 μm. (**d**) PARP cleavage is blocked by PAF and 5-HT receptor antagonists. Keratinocytes were pretreated with PCA 4248 (50 μM) or 1-NPZ (25 μM), washed, and then exposed to 200 J m^{-2} of UVB radiation. Total cellular protein was isolated 1 and 24 hours post irradiation. Cleaved PARP was measured by western blotting. Equal gel loading was confirmed by staining with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific antibody.

et al., 1989; Kripke *et al.*, 1992; Vink *et al.*, 1997). Compelling data also exist showing that *cis*-UCA is a UV photoreceptor (De Fabo and Noonan, 1983) and that neutralizing its function will also impair photocarcinogenesis (Beissert *et al.*, 2001) and block the induction of immune suppression (Moodycliffe *et al.*, 1996; Walterscheid *et al.*, 2006). Moreover, injecting PAF receptor antagonists into UV-irradiated mice blocks both immune suppression and photocarcinogenesis (Walterscheid *et al.*, 2002; Sreevidya *et al.*, 2008). Conventional wisdom suggests that the reason

anti-*cis*-UCA antibody, PAF receptor, and 5-HT receptor antagonists block photocarcinogenesis is by blocking immune suppression (as illustrated in Figure 1). Undoubtedly, blocking immune suppression is contributing to some of the anticancer effects, but our findings illustrate a previously unreported and as-yet unappreciated mechanism to describe the anticancer effects previously described. We show that blocking the binding of *cis*-UCA to its receptor (5-HT_{2A}), or blocking the binding of PAF to its receptor, agents that reverse UV-induced immune suppression and photocarcinogenesis, also modulate DNA repair. Both UV-induced photoproducts (CPD and 6–4 photoproducts) were removed following treatment with PAF and 5-HT receptor antagonists. As this is an NER-dependent mechanism, we measured UDS. Treatment with the PAF and 5-HT receptor antagonists accelerated UDS, indicating that these agents accelerated NER. Failure of the PAF and 5-HT receptor antagonists to accelerate DNA repair in *XPA*−/− mice further supports our hypothesis that these agents affect NER. The implication of these findings is that UV-induced immunosuppressive agents, such as PAF and *cis*-UCA, inhibit DNA repair *in vivo*. It is interesting to note that Yarosh *et al*. (2005) showed that two classic immunosuppressive drugs, cyclosporine and tacrolimus, also inhibit the removal of CPD in UV-irradiated keratinocytes.

Our findings also indicate that treating mice with *cis*-UCA, PAF, and 5-HT induces ROS, leading to oxygen radical formation and DNA damage, as evidenced by 8-oxo-dG formation. The induction of ROS by PAF and 5-HT, which are well-known inflammatory mediators, is documented (Bussolati *et al*., 1998; Breard *et al*., 2007). Although others have suggested that it is theoretically possible for *cis*-UCA to induce ROS (Shen and Ji, 2008), here we provide direct evidence for *cis*-UCA-induced ROS and DNA damage. In addition, we provide data showing that blocking the binding of *cis*-UCA and PAF to their receptors blocks ROS and 8-oxo-dG formation. These findings suggest that agents used here that reverse UV-induced immune suppression and photocarcinogenesis may do so by regulating DNA repair.

Kripke and colleagues clearly showed the role of UV-induced CPD formation in activating immune suppression. However, it is equally important to remember that other types of DNA damage, particularly double-strand breaks, will also induce immunosuppression and the production of immune suppressive cytokines *in vivo* (O'Connor *et al*., 1996; Nishigori *et al*., 1998). We suggest that this is one pathway by which *cis*-UCA and PAF are activating immune suppression, in the absence of the typical UV-induced DNA damage (Figure 1). 8-oxo-dG formation is indicative of oxidative DNA damage. Treating keratinocytes, or skin with PAF, *cis*-UCA, or 5-HT activates DNA damage, as evidenced by the upregulation of 8-oxo-dG. Moreover, PAF and 5-HT receptor antagonists decrease 8-oxo-dG formation *in vivo*. Our data indicate that the PAF and 5-HT receptor antagonists can work at two levels to repair DNA lesions. We see accelerated repair of both UV-induced photoproducts (CPD and 6–4 photoproducts) and reduced numbers of ROS-induced lesions (8-oxo-dG formation) in mice treated with PAF and 5-HT receptor antagonists. Indeed, these two observations may be related. Others have shown that ROS can adversely affect NER. Langie *et al*. found that NER capacity as well as the expression of some of the genes involved in NER is modulated by oxidative stress. Specifically, they noted an inhibition of NER after exposure to H_2O_2 (Langie *et al*., 2007). It may be possible that UV-induced *cis*-UCA and PAF are contributing to immune suppression and photocarcinogenesis not only by inducing DNA damage through the production of reactive oxygen but also by inhibiting NER and allowing for the persistence of CPD and 6–4 photoproducts through an ROS-dependent mechanism. This may help explain why these seemingly disparate agents block both UV-induced and *cis*-UCA-induced immune suppression.

Interleukin-12 is a classic immune modulatory cytokine that has been shown to reverse UV-induced immune suppression (Schmitt *et al*., 1995). Schwarz *et al*. (2002, 2005) showed that IL-12 treatment reverses UV-induced apoptosis and immune suppression, in part, by activating DNA repair. Our findings are reminiscent of the dual effects of IL-12. For example, blocking PAF from binding to its receptor restores immune competence, by suppressing prostaglandin E_2 and IL-10 production (Walterscheid *et al*., 2002), and as shown here, PAF receptor antagonists accelerate DNA repair. The implication of this finding is that inflammatory mediators, such as PAF, depress DNA repair, thus providing another mechanistic link between inflammation and carcinogenesis. Supporting this idea are the observations that PAF receptor binding has been implicated in a variety of cancers, including melanoma (Melnikova *et al*., 2006), ovarian (Aponte *et al*., 2008), breast (Montrucchio *et al*., 1998), brain (Denizot *et al*., 2006), and leukemia (Denizot *et al*., 2004).

In summary, our data show that agents that block UV-induced immune suppression do so by modulating DNA repair. Damage caused by a direct result of UV radiation (CPD and 6–4 photoproducts), as well as damage due to UV-induced ROS (8-oxo-dG), was reduced when the binding of PAF to its receptor and the binding of *cis*-UCA to its receptor were blocked. Moreover, treating keratinocytes with inflammatory mediators induced DNA damage. Impaired DNA repair has serious consequences in humans, as evidenced by the clinical features associated with xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (van Steeg and Kraemer, 1999). The findings reported here confirm the important role that DNA damage and DNA repair have in UV-induced immune suppression. Further, they provide a mechanism describing how different UV-induced mediators, such as PAF and *cis*-UCA, can regulate DNA repair, which affects immune suppression and contributes to UV-induced carcinogenesis.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the NCI-Frederick Cancer Research Facility, (Frederick, MD). *XPA*−/− mice (Nakane *et al*.,

1995) were bred at the Kobe University Graduate School of Medicine (Kobe, Japan). All animals were maintained with alternating 12-hour light and dark cycles and controlled temperature and humidity in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee and the Animal Ethics Committee of the Kobe University Graduate School of Medicine approved all the animal procedures described here.

Reagents

cPAF, PCA-4248, 1-NPZ, and ketanserin were purchased from Biomol Research Labs (Plymouth Meeting, PA). Dichlorofluorescein diacetate, OPD Peroxidase substrate, and *cis*-UCA were purchased from Sigma-Aldrich (St Louis, MO). The murine keratinocyte cell line Pam 212 was a gift from Dr Stuart Yuspa, National Cancer Institute, Bethesda, MD. Tissue culture medium was obtained from Gibco BRL (Grand Island, NY). Biotin-SP-conjugated goat anti-mouse immunoglobulin and peroxidase-conjugated streptavidin were purchased from Jackson ImmunoResearch Labs (West Grove, PA). An antibody specific for cleaved PARP was purchased from Cell Signaling Technology (Danvers, MA). TUNEL-positive cells were detected using a commercial kit provided by Promega (Madison, WI).

Radiation source

Mice were exposed to UV radiation supplied by a 1000 W xenon UV solar simulator (Oriel, Stratford, CT) (Sreevidya *et al.*, 2008). To irradiate keratinocytes in culture, a single FS-40 sunlight was used (Rivas and Ullrich, 1992). The intensity and spectral output of the radiation sources were measured with an Optronics model OL-754 scanning spectrophotometer (Optronics Labs, Orlando, FL).

UDS

Pam 212 cells were treated with 50 μM of PCA-4248 and 25 μM of 1-NPZ for 30 minutes at 37°C. These doses were chosen on the basis of previous studies (Walterscheid *et al.*, 2002, 2006; Sreevidya *et al.*, 2008). The cells were washed extensively, overlaid with phosphate-buffered saline, and then exposed to UV radiation (200 J m^{-2}). The cells were incubated for 3 hours at 37°C with 10 μM of BrdU. The cells were then fixed in methanol, and UDS was analyzed as described (Nakagawa *et al.*, 1998). UDS was visualized by counting punctuated spots in the nuclei, in contrast to the uniformly intense staining found in ordinary DNA synthesis. The grains per nuclei counted in at least 50 cells undergoing UDS were recorded. The percentage of cells undergoing UDS was calculated by counting at least 10 fields per sample.

Measurement of CPD, 6–4 PP, and 8-oxo-dG

C57BL/6 mice were exposed to 2 kJ m^{-2} of UVB radiation and injected i.p. with 1 μM of PCA-4248 or 1 μM of 1-NPZ. These doses were chosen on the basis of previous studies (Walterscheid *et al.*, 2002, 2006; Sreevidya *et al.*, 2008). Skin sections were collected at various times post exposure and fixed as previously described (Sreevidya *et al.*, 2008). CPD was detected using a monoclonal anti-thymine dimer (clone H3, Sigma-Aldrich). 6–4 photoproducts were detected using monoclonal antibody, D195–1 clone 64M2 (MBL International, Woburn, MA). 8-oxo-dG was detected using monoclonal antibody N45.1 (MBL International). Genomic DNA was purified using DNeasy kit (Qiagen, Valencia, CA). CPD and 6–4 photoproducts were also measured by ELISA (MBL International). An ELISA to detect 8-oxo-dG was acquired from Genox (Baltimore, MD).

Measurement of ROS

Pam 212 cells were treated with 50 μM of PCA-4248 and 25 μM of 1-NPZ. After 30 minutes, the cells were washed, overlaid with phosphate-buffered saline, and exposed to 200 J m^{-2} of UVB radiation. Alternatively, some cells were treated with 10 μM of cPAF, 50 μM of 5-HT, or 50 μM of *cis*-UCA. The cells were washed, trypsinized, and resuspended in 5% fetal bovine serum/Hank's balanced salt solution. Dichlorofluorescein diacetate (2 μM) was added, and after 1 hour at room temperature, fluorescence was measured by flow cytometry (FACS Caliber, Becton Dickenson, San Jose, CA). Alternatively, 1×10^5 Pam 212 cells were added to Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY) and cultured overnight. The cells were treated with PAF and 5-HT receptor antagonists, and then exposed to UVB radiation, as described above. A total of 5 μM of aminophenyl fluorescein and 5 μM of hydroxyphenyl fluorescein (Sigma-Aldrich) were added. After 30 minutes, the monolayers were washed and fluorescence was detected by microscopy.

Immune suppression

Suppression of the induction of a delayed-type hypersensitivity reaction, as described in detail elsewhere (Nghiem *et al.*, 2002), was used to measure the effect of UV, *cis*-UCA, 5-HT, and PAF on the immune response. C57Bl/6 mice were first exposed to 15 kJ m^{-2} of UVB radiation and immunized 5 days later with formalin-fixed *Candida albicans*. Delayed-type hypersensitivity, as determined by an increase in footpad swelling in response to a challenge with *C. albicans*-specific antigen (Alerchek, Portland, ME), was measured 10 days post immunization. T4N5-containing liposomes were a gift from Dan Yarosh (AGI-Dermatics, Freeport, NY) and were used as previously described (Nishigori *et al.*, 1996). Mice were injected i.p. with 5 μM *cis*-UCA (Walterscheid *et al.*, 2006), 250 pM of 5-HT (Walterscheid *et al.*, 2006), and 500 pM of cPAF (Walterscheid *et al.*, 2002) to induce immune suppression, and 10 μg of anti-*cis*-UCA antibody (Moodycliffe *et al.*, 1996), 1 μM of PCA-4248 (Walterscheid *et al.*, 2002), and 1 μM of ketanserin (Walterscheid *et al.*, 2006) to block immune suppression. Anti-*cis*-UCA, PCA-4248, and ketanserin were injected i.p. immediately following UV exposure. There were at least five mice per group. The mean footpad thickness for the group ± the SEM was also calculated. A statistical difference between the positive control and the experimental groups was determined using a one-way ANOVA followed by the Dunnett's Multiple Comparison test (GraphPad Prism Software, GraphPad, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

SSC and AF contributed equally to this work. This work was funded by grants from the National Cancer Institute (CA112660 & CA131207). CSS was supported by an NCI training grant (T32-CA-09598-15). The animal, histology, and flow cytometry facilities at UTMDACC were supported in part by an NCI Cancer Center Support Grant (CA16672). We thank Dr Mary Norval for the gifts of *cis*-UCA and anti-*cis*-UCA monoclonal antibody.

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